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Detection of *Escherichia coli* O157:H7 in Food Using Real-Time Multiplex PCR Assays Targeting the stx_1, stx_2, wzy_{O157}, and the $fliC_{h7}$ or *eae* Genes

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Abstract *Escherichia coli* O157:H7 is an important foodborne pathogen, and foods of bovine origin and fresh produce have been linked to outbreaks. Real-time multiplex PCR assays were developed to detect *E. coli* O157:H7 in different foods. Apple cider and raw milk (25 ml) and ground beef and lettuce (25 g) were inoculated with 2 or 20 colony-forming units (CFU) of *E. coli* O157:H7 380-94 and subjected to enrichment in RapidChek *E. coli* O157:H7 broth at 42°C. One milliliter of the enrichments was removed at 8 and 20 h, and following DNA extraction, real-time multiplex PCR assays targeting the stx_1, stx_2, and wzy_{O157} genes in combination with probes and primers targeting either the $fliC_{h7}$ or the *eae* genes were performed using OmniMix HS beads and the SmartCycler. The sensitivity of the real-time multiplex PCR assay was about 225 CFU/PCR. *E. coli* O157:H7 was detected (fluorescent signal generated for all gene targets) in apple cider, raw milk, lettuce and ground beef samples inoculated with 2 or 20 CFU/g or 25 ml after both 8 and 20 h of enrichment. Enrichments of uninoculated food samples were negative using the multiplex PCR targeting the stx_1, stx_2, wzy_{O157}, and *eae* genes; however, using the assay targeting the stx_1, stx_2, wzy_{O157}, and $fliC_{h7}$ gene combination, a positive result was always obtained for the $fliC_{h7}$ gene using uninoculated ground beef enrichments. Use of other primer sets targeting the $fliC_{h7}$ gene gave similar results. The real-time multiplex PCR assays targeting the stx_1, stx_2, *eae*, and wzy_{O157} or the $fliC_{h7}$ genes are sensitive and specific and can be used for the detection of *E. coli* O157:H7 in food, except that the $fliC_{h7}$ gene may not be a suitable target for the detection of *E. coli* O157:H7 in ground beef.

Keywords Food · Detection · *E. coli* O157:H7 · Multiplex PCR · Real-time

Introduction

Escherichia coli O157:H7 is an important foodborne pathogen associated with sporadic cases and outbreaks of bloody diarrhea, which can progress to a serious complication known as hemolytic uremic syndrome, particularly in the young and the elderly. Cattle are the most important reservoir for *E. coli* O157:H7; however, the pathogen has been isolated from other animals, including deer, goats, horses, dogs, and pigs (Smith and Fratamico 2005). Produce can become contaminated in the field from animals, manure, or contaminated irrigation water. Outbreaks associated with *E. coli* O157:H7 have been linked to food of bovine origin, including ground beef and raw milk hard cheese, and also to produce, apple cider, and drinking and recreational water (Anonymous 2000; Hilborn et al. 2000; Olsen et al. 2002; Sivapalasingam et al. 2004; Honish et al. 2005; Rangel et al. 2005; CDC 2006; Wendel et al. 2009)

Rapid and reliable methods for the detection of *E. coli* O157:H7 in different food products are needed to ensure food safety. The combination of rapidity, good sensitivity and specificity, and ease of performance has made PCR

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technology an appealing alternative to culture-based and immunological-based methods for pathogen detection in foods. However, since the PCR is often inhibited by components in food samples and other complex matrices, sample preparation methods, such as filtration or DNA extraction, are routinely performed on food enrichments before target DNA amplification (Rudi et al. 2002). Real-time PCR, which involves the use of fluorescent probes or DNA-binding dyes in the reaction, offers several advantages over traditional end-point PCR methods. The closed-tube detection format lowers the potential for carryover contamination and false positive results, there is a shorter analytical turnaround time and generally higher sensitivity and specificity than end-point assays, and there is no need for post-PCR processing steps to detect the PCR product/s.

Shiga toxins [Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2)] are the most critical virulence factors associated with the clinical characteristics of *E. coli* O157:H7 infection; however, other important virulence genes include the *eae* gene (encoding the intimin outer membrane protein) located in the locus of enterocyte effacement (Gyles 2007). PCR assays targeting the Shiga toxin genes have been developed for the detection of *E. coli* O157:H7 (Fode-Vaughan et al. 2003); however, these assays are not specific for this pathogen, since other Shiga toxin-producing *E. coli* will also be detected. More definitive identification of *E. coli* O157:H7 can be accomplished by amplification of pathogen-specific sequences in addition to the *stx* genes using multiplex PCR assays. Advantages of multiplex PCR assays, in which two or more DNA regions are co-amplified in one reaction, are lower cost and less time to obtain results. Real-time multiplex assays are performed using multiple dyes with distinct emission wavelengths, and a number of multiplex real-time PCR assays have been described for the detection and quantification of pathogens in food (Hong et al. 2007; Wang et al. 2007; Omiccioli et al. 2009). In the present study, real-time PCR assays using two combinations of primers and probes were designed for the detection of *E. coli* O157:H7 in apple cider, raw ground beef, lettuce, and raw milk. The first assay targeted the wzy_{O157} gene in the *E. coli* O157 O antigen gene cluster, which encodes for the O antigen polymerase, the stx_1 and stx_2 genes, and the gene encoding the H7 flagellin ($fliC_{h7}$). The second assay targeted the wzy_{O157}, stx_1 and stx_2, and the *eae* genes.

Materials and Methods

Bacterial Strains

The bacteria used in this study included *E. coli* O157:H7 strains 380-94 (Food Safety and Inspection Service, salami, outbreak strain), C984 [Centers for Disease Control and Prevention (CDC), Atlanta, GA, clinical isolate], B1409-C1 (CDC, clinical isolate), *E. coli* standard H types (H1 through H56, except H13, H22 and H50 that are cancelled H types), and *E. coli* serogroups O1 through O173, but excluding O14, O31, O47, O67, O72, O93, O94, and O122 strains, since these serogroup designations have been cancelled, and OX3, OX6, OX7, OX9, OX10, OX13, OX18, OX19, OX21, OX23, OX25, OX28, OX38, and OX43 strains. Twenty-five non-O157 Shiga toxin-producing *E. coli* and 20 non-*E. coli* bacteria, including *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus aerogenes*, *Enterococcus faecalis*, *Hafnia alvei*, *Klebsiella pneumonia*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella enterica* subsp. Anatum, Arizona, Choleraesuis, Enteritidis, and Typhimurium, *Serratia marcescens*, *Shigella boydii*, *Staphylococus aureus*, *Vibrio cholerae*, and *Yersinia enterocolitica* were also tested. The bacteria were routinely grown in tryptic soy broth (Becton Dickinson, Sparks, MD, USA) or on tryptic soy agar (TSA) plates.

Multiplex PCR Assays and Determination of PCR Sensitivity

Primers and probes were designed using the Primer3 software program (http://frodo.wi.mit.edu/primer3/) and are shown in Table 1. *E. coli* O157:H7 strains 380-94 (carries stx_1 and stx_2), C984 (carries stx_1), and B1409-C1 (carries stx_2), and other bacteria listed above were grown on TSA plates overnight, and DNA was extracted using the PrepMan Ultra reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions from colonies resuspended in 1 ml of sterile water and then subjected to centrifugation at 16,000×*g* for 2 min. Approximately 150 µl of the supernatant containing the DNA were collected and either used immediately or stored at −20°C until testing.

To determine the sensitivity of the multiplex PCR assays, 1 ml of an overnight culture containing about 10^9 CFU/ml was subjected to DNA extraction using PrepMan Ultra, and then tenfold serial dilutions of the DNA were made. To determine the CFU per milliliter of *E. coli* O157:H7, the bacterial suspension was diluted and plated onto TSA, and colonies were counted after overnight incubation. The PCR was performed using OmniMix HS Reagent beads (Fisher Scientific, Pittsburgh, PA, USA) that were reconstituted as directed by the manufacturer and consisted of 1.5 U TaKaRa hot start *Taq* polymerase, 200 µM dNTP, 4 mM $MgCl_2$, and 25 mM HEPES, pH 8.0. Two multiplex PCR assays were performed. For assay 1, the target sequences were the wzy_{O157}, stx_1, stx_2, and *eae* genes (sizes of PCR products were 112, 200, 140, and 167 bp, respectively), and for

assay 2, the target genes were wzy_{O157}, stx_1, stx_2, and $fliC_{h7}$ (sizes of products were 112, 200, 140, and 247 bp, respectively). For assay 1, 0.1 μM of primers for *eae*, stx_1, and stx_2, 0.3 μM of primers for wzy_{O157}, and 0.125 μM of each of the probes were used (Table 1). Whereas for assay 2, 0.25 μM of each of the primers and 0.125 μM of the probes were used. To 22.5 μl of the reaction mixture, 2.5 μl of template DNA were added. The PCR was performed using the SmartCycler (Cepheid, Sunnyvale. CA, USA), and the cycling protocol consisted of 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 50 s. The standard curves were generated by plotting the log value of the calculated CFU per reaction corresponding to the diluted DNA samples versus the threshold cycle (Ct), which is defined as the cycle where the fluorescence signal crosses a defined threshold (set at 30 fluorescence units).

The detection limit was also determined by adding dilutions of *E. coli* O157:H7 to food enrichments. Dilutions were made from an overnight culture of *E. coli* O157:H7 380-94, and 100 μl were added to 900 μl aliquots of uninoculated ground beef, raw milk, lettuce, and apple cider enrichments for final concentrations ranging from 10^8 to 10^1 CFU/ml. The DNA was extracted using the PrepMan Ultra reagent as described above, and 2.5 μl of template DNA were used in the multiplex PCR assays. The limit of detection of the assays was determined as an average of the minimum number of CFU per PCR reaction required for a positive Ct value over the threshold for all of the fluorescence signals.

PCR Specificity Testing

The primers targeting the *E. coli* O157 *wzy* gene were tested against the *E. coli* reference standard strains, *E. coli* O157:H7 strains, and the non-*E. coli* bacteria listed above using only the primers targeting the wzy_{O157} gene in the PCR. The specificity of the $fliC_{h7}$ primers was tested using *E. coli* strains belonging to all of the known H types, as described above, using only the primers targeting $fliC_{h7}$. The reaction and cycling conditions were as described above for the multiplex PCR. The *eae*, stx_1, and stx_2 primers were tested for specificity against 25 non-O157 Shiga toxin-producing *E. coli* and 20 non-*E. coli* bacteria.

Table 1 Oligonucleotide primers and probes used in the real-time multiplex PCR assays for detection of *E. coli* O157:H7

	Primer sequences 5′ to 3′	PCR product size	GenBank accession no.
Primer sets			
EAE2325-F	GTAAGTCTCAAACGCAAGCAACCAC	167	X60439
EAE2491-R	AACCTTGTTGTCAATTTTCAGTTCATCA		
FLICH71068-F	TACCATCGCAAAAGCAACTCC	247	U47614
FLICH71314-R	GTCGGCAACGTTAGTGATACC		
Stx1-418-F	CTCGACTGCAAAGACGTATG	200	M16626
Stx1-617-R	TTCGTTCAACAATAAGCCGTAGATT		
Stx2-314-F	ACGATAGACTTTTCGACCCAACAA	140	X07865
Stx2-453-R	AAATAACTGCCCGGTGGGGT		
WzyO157-528-F	CCTGTCAAAGGATAACCGTAATCC	112	AF061251
WzyO157-639-R	TTGTTCTCCGTCTTGTCCTAAACT		
LuxS-F	AGCGATCAAAGCAAAATTCC	87	DQ143940
LuxS-R	GGCAATTTGTTTGGCTTCAT		
PUC19-F	GCAGCCACTGGTAACAGGAT	118	L09137
PUC19-R	GCAGAGCGCAGATACCAAAT		
16SrRNA-IC-F	CCTCTTGCCATCGGATGTG	99	GU352926
16SrRNA-IC-R	GGCTGGTCATCCTCTCAGACC		
Probes			
EAE-P	5′-TET-CGGCGGAACTGGAAGTTAGTGTTATCGTCG-3′-BHQ1		X60439
FLICH7-P	5′-TET-CGGCTGCCGCGACATCTTCAAT-BHQ1		U47614
Stx1-P	5′-Texas Red-CGCTGAATGTCATTCGCTCTGCA-BHQ2		M16626
Stx2-P	5′-Texas Red-AACAGACACCGATGTGGTCCCCTGAGAT-BHQ2		X07865
WzyO157-P	5′-FAM-AAAACAACGAGCATACAACCCCTACCAAT-BHQ1		AF061251
LuxS IC-P	5′-Cy5-TTAATATCTATCAATGCGGAACTTGCGCAA-3′-BHQ2		DQ143940
PUC19-P	5′-Cy5-AGAGCGAGGTATGTAGGCGG-3′-BHQ2		L09137
16SrRNA-IC-P	5′-Cy5-GTGGGGTAACGGCTCACCTAGGCGAC-3′-BHQ2		GU352926

Inoculation and Enrichment of Food Samples

Apple cider, ground beef (15% fat), and lettuce were purchased from local supermarkets, and the raw milk was obtained from a local agricultural high school. An overnight culture of *E. coli* 380-94 was serially diluted in 0.1% peptone and then plated onto TSA to determine the CFU per milliliter. In a 500-ml flask, 1 ml of the dilution with about 2 CFU/ml and a dilution with about 20 CFU/ml was inoculated into 25 g or 25 ml of the food samples, and 225 ml of RapidChek *E. coli* O157:H7 enrichment broth (REB; Strategic Diagnostics, Inc., Newark, DE, USA) were added. Since 2 CFU is a very low inoculum level and to ensure that at least one sample would receive 2 CFU, in each experiment, two samples were inoculated with 1 ml of the dilution containing about 2 CFU, and one sample was inoculated with about 20 CFU. Each experiment also included uninoculated samples. The flasks were incubated at 42°C at 100 rpm. After 8 and 20 h, 10 ml of the enrichments were collected; dilutions were made in 0.1% peptone and plated onto CHROMagar O157 (Dynal Biotech, Inc., Lake Success, NY, USA) and were used for the multiplex PCR assays.

DNA Extraction and Detection of *E. coli* O157:H7 in Food Using Real-Time Multiplex PCR Assays

One milliliter of the enrichments was subjected to DNA extraction using the PrepMan Ultra reagent as described earlier, and 2.5 μl of the DNA were used as template for the real-time multiplex PCR assays performed as described above using OmniMix HS beads and the primers and probes shown in Table 1.

Use of Non-competitive Internal Controls

Three non-competitive internal controls used for preventing reporting of false negative results due to the presence of PCR inhibitors in food samples were evaluated in the multiplex PCR assays. pYH201 containing the *Campylobacter jejuni luxS* gene was constructed by Dr. Yiping He (USDA, Eastern Regional Research Center). Briefly, an 87-bp product was amplified from *C. jejuni* 81-176 chromosomal DNA with the LuxS primers shown in Table 1. The PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The resulting plasmid, pYH201, was linearized by digestion with *Hin*dIII, and the concentration of the linearized plasmid DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To determine the optimal concentration of the internal control for multiplex PCR, serial dilutions of linearized pYH201 were made, and 1 μl of the dilutions was added to the PCR containing 2.5 μl of water or an 8-h uninoculated ground beef enrichment so that the final concentration of pYH201 was 10^1–10^7 copies per PCR. The concentration that resulted in a Ct of about 22–24 was 10^5 copies (2 pg); therefore, this concentration of pYH201 (1 μl) and also 0.3 μM of LuxS primers (LuxS-F and LuxS-R) and 0.125 μM of LuxS probe (Table 1) labeled with Cy5 were included in all the multiplex PCR assays. The fluorescent signal generated from the four probes was read in four separate channels in the SmartCycler. The second internal control that was evaluated consisted of pUC19 (New England Biolabs, Ipswich, MA, USA) and primers and Cy5-labeled probe targeting the pMB1 replicon *rep* (Fricker et al. 2007) (Table 1). Primers were used at a concentration of 0.25 μM and the probe at 0.2 μM in the multiplex PCR assays. pUC19 was linearized with *Eco*RI and used in the PCR in the same way as pYH201. The optimal concentration of linearized pUC19 was 10^4 copies/PCR (Ct about 25–27), and the size of the PCR product was 118 bp. The third internal control system targeted the 16S rRNA gene (gamma-proteobacteria) as previously described by Fratamico et al. (2009); however, a probe labeled with Cy5 was used (Table 1). The primers were used at a concentration of 0.1 μM and the probe at 0.03 μM. The Cts for the 16S rRNA internal control were in the range of about 18–24, and the size of the PCR product was 99 bp.

Results and Discussion

Two real-time multiplex PCR assays were used for the detection of *E. coli* O157:H7 in artificially inoculated ground beef, lettuce, raw milk, and apple cider. Primers targeting the $fliC_{h7}$ and wzy_{O157} genes were specific for *E. coli* O157 and strains possessing the H7 antigen and the O157 antigen, respectively, and no non-specific results were obtained by the PCR with non-H7 and non-O157 *E. coli* strains or non-*E. coli* bacteria. Likewise, only *E. coli* possessing the stx_1 and stx_2 genes were positive using the multiplex PCR assays, and only *E. coli* O157:H7 and O55:H7, which harbors and *eae* homologue of $eae_{O157:H7}$ (Sharma and Dean-Nystrom 2003), strains were positive for *eae*. The PCR products obtained using singleplex and multiplex PCR assays are shown in Fig. 1a and b.

Since the multiplex PCR assays employ two sets of primers for the *stx* genes, one targeting stx_1 and one targeting stx_2, but the probes are both labeled with the Texas Red dye, the possibility that strains that harbor both stx_1 and stx_2 will give a stronger signal than strains that possess only one of the toxins was explored. Therefore, three *E. coli* O157:H7 strains, 380-94 (stx_1 and stx_2), C984

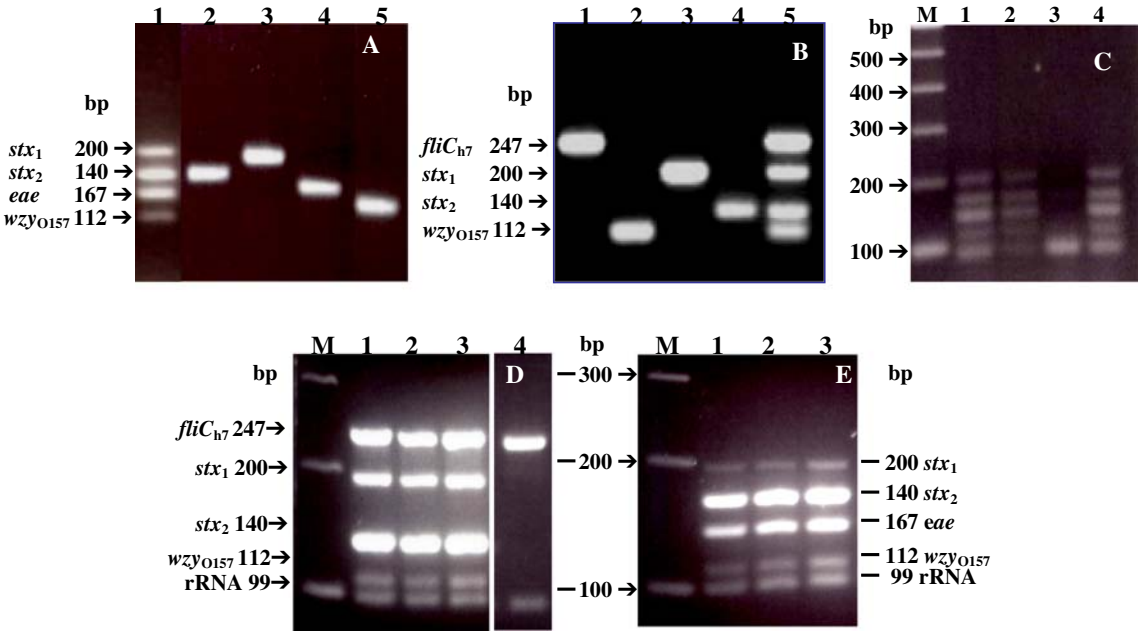

Fig. 1 Agarose gels showing results of the multiplex PCR assays. **a** *Lane 1* Multiplex PCR targeting stx_1, stx_2, *eae*, and wzy_{O157} genes simultaneously in one reaction tube; *lanes 2–5* singleplex PCR assays targeting the stx_2, stx_1, *eae*, and wzy_{O157} genes, respectively. **b** *Lanes 1–4* Singleplex PCR assays targeting the $fliC_{h7}$, wzy_{O157}, stx_1, stx_2, genes, respectively; *lane 5* multiplex PCR targeting all four genes in a single reaction tube. **c** Multiplex PCR assays (*eae*, wzy_{O157}, stx_1, and stx_2) of enrichments of lettuce samples including the internal control targeting *luxS* (87 bp). *Lane M* 100-bp ladder (Invitrogen); PCR products from DNA from ground beef samples inoculated with 2 CFU and after 8 h enrichment (*lane 1*), 20 CFU after 8 h enrichment (*lane 2*), uninoculated after 20 h enrichment (*lane 3*), and 2 CFU after 20 h enrichment (*lane 4*). Five products are visible: *luxS* internal control (87 bp), wzy_{O157} (112 bp), stx_2, (140), *eae* (167 bp), and stx_1 (200 bp). **d** Multiplex PCR assays ($fliC_{h7}$, wzy_{O157}, stx_1, and stx_2) of enrichments of lettuce samples using the rRNA gene as the internal control. *Lane M* 100-bp ladder; PCR products from DNA from lettuce samples inoculated with 2 CFU and after 8 h enrichment (*lane 1*), 20 CFU after 8 h enrichment (*lane 2*), and 2 CFU after 20 h enrichment (*lane 3*). *Lane 4* Uninoculated ground beef enrichment showing the rRNA internal control band and the $fliC_{h7}$ gene product. **e** Multiplex PCR assays (*eae*, wzy_{O157}, stx_1, and stx_2) of enrichments of lettuce samples using the rRNA gene as the internal control. *Lane M* 100-bp ladder; PCR products from DNA from lettuce samples inoculated with 2 CFU and after 8 h enrichment (*lane 1*), 20 CFU after 8 h enrichment (*lane 2*), and 2 CFU after 20 h enrichment (*lane 3*)

(stx_1), and B1409-C1 (stx_2) were tested using the multiplex PCR assays to determine if the presence of only one or both Shiga toxin genes influenced the Ct values. Data from the standard curves that were generated showed that similar Ct values for *stx* were obtained with all three strains (data not shown).

Standard curves were generated to determine the detection limit of the assays. The plots of the log of the initial target copy number for a set of standards (diluted *E. coli* O157:H7 380-94, C984, and B1409-C1 DNA) versus Ct were linear with R^2 values greater than 0.99 over six orders of magnitude (10^4–10^9CFU/ml) (data not shown). The sensitivity of the multiplex PCR assays, the lowest concentration in which a positive fluorescent signal was generated with all four probes, was about 225 CFU/PCR (2.5 μl of template DNA). The PCR assay was also performed using 1 μl of template DNA, and generally, the same results were obtained as with 2.5 μl of template; therefore, the detection limit may be lower than 225 CFU/PCR. The same results were obtained using dilutions of *E. coli* O157:H7 DNA made in sterile water and using DNA extracted from uninoculated food enrichments to which dilutions of *E. coli* O157:H7 were added. Ibeweke et al. (2002) obtained a detection limit of $\geq 3.5 \times 10^4$CFU/g for *E. coli* O157:H7 in soil, manure, feces, and wastewater samples using a real-time multiplex PCR assay, and their sensitivity increased to 10 CFU/g with a 16-h enrichment. In the current work, the sensitivity of assays 1 and 2 was ≤2 CFU/25 g or milliliter of sample after 8 h of enrichment.

The Ct values obtained with real-time multiplex PCR assays 1 and 2 for detection of *E. coli* O157:H7 in REB enrichments of artificially inoculated apple cider, ground beef, lettuce, and raw milk samples are shown in Table 2. All results were positive for samples inoculated with about 2 and about 20 CFU/25 g or 25 ml of food after 8 and 20 h of enrichment. For assay 1, all of the uninoculated controls were negative (data not shown). Previous work showed that, overall, a higher sensitivity for the detection of non-stressed, cold-, and freeze-stressed *E. coli* O157:H7 by culture, an immunoassay, and by a PCR assay was achieved using REB compared to R&F *E. coli* O157:H7 enrichment broth, or modified *E. coli* broth with novobiocin (Fratamico

Table 2 Cycle threshold (Ct) values obtained with real-time multiplex PCR assays 1 and 2 using 8- and 20-h food enrichments

Sample type, inoculum level and enrichment time (cycle threshold value ± SD)	Target genes					
	wzy_{O157}	*eae*	stx_1-stx_2	wzy_{O157}	$fliC_{h7}$	stx_1-stx_2
	Assay 1 (Ct ± SD)			Assay 2 (Ct ± SD)		
Ground beef						
Uninoculated (8 h)					23.8±0.7[a]	
2 CFU/25 g (8 h)	25.6±0.9	23.7±0.5	23.7±0.9	26.3±0.8	24.6±0.3	23.6±0.5
2 CFU/25 g (8 h)	26.9±0.9	24.6±0.9	23.3±0.9	26.5±0.6	24.2±0.5	24.3±0.4
20 CFU/25 g (8 h)	22.3±0.8	24.6±0.9	20.2±0.8	23.4±0.5	20.5±0.7	21.5±0.7
Uninoculated (20 h)					20.3±0.6[a]	
2 CFU/25 g (20 h)	26.2±0.9	25.0±0.4	24.8±0.3	24.9±0.2	21.2±0.7	22.0±0.6
2 CFU/25 g (20 h)	26.5±0.6	24.4±0.9	24.4±0.2	24.9±0.5	21.2±0.6	22.2±0.5
20 CFU/25 g (20 h)	26.3±0.4	23.2±0.8	23.1±0.1	23.6±0.8	18.0±0.7	18.1±0.5
Raw milk						
2 CFU/25 g (8 h)	26.4±0.5	25.2±0.9	24.7±0.3	27.4±0.6	25.9±0.5	26.8±0.8
2 CFU/25 g (8 h)	25.6±0.6	24.4±0.4	24.4±0.5	26.8±0.7	25.1±0.6	25.9±0.9
20 CFU/25 g (8 h)	22.5±0.6	21.1±0.5	21.5±0.3	23.5±0.3	22.1±0.7	22.8±0.4
2 CFU/25 g (20 h)	24.7±0.2	24.4±0.4	23.6±0.1	28.1±0.9	27.9±0.7	26.4±0.4
2 CFU/25 g (20 h)	25.1±0.8	24.1±0.8	24.1±0.9	27.0±0.3	25.9±0.9	25.3±0.6
20 CFU/25 g (20 h)	22.2±1.0	20.9±0.3	21.6±0.9	25.4±0.8	22.9±0.7	21.6±0.3
Lettuce						
2 CFU/25 g (8 h)	20.9±0.8	17.7±0.5	19.3±0.2	22.9±0.6	23.4±0.4	21.0±0.6
2 CFU/25 g (8 h)	21.7±0.9	18.4±0.3	20.1±0.2	23.8±0.7	24.4±0.5	21.4±0.7
20 CFU/25 g (8 h)	17.4±0.7	15.0±0.8	15.9±0.4	22.1±0.7	20.2±0.5	19.4±0.4
2 CFU/25 g (20 h)	16.8±0.2	15.5±0.6	15.3±0.0	20.4±0.4	21.1±0.7	17.4±0.4
2 CFU/25 g (20 h)	17.4±0.9	16.0±0.9	15.5±0.1	21.4±0.9	18.7±0.2	16.0±0.3
20 CFU/25 g (20 h)	17.4±1.0	15.7±0.3	15.5±0.2	18.2±0.6	18.0±0.4	15.6±0.6
Apple cider						
2 CFU/25 g (8 h)	20.2±0.7	17.3±0.6	18.1±0.7	22.9±0.7	23.8±0.5	21.1±0.4
2 CFU/25 g (8 h)	21.5±0.8	18.0±0.5	19.3±0.1	23.1±0.5	24.4±0.7	20.7±0.3
20 CFU/25 g (8 h)	18.8±1.0	16.0±0.6	16.9±0.3	19.9±0.6	22.1±0.5	19.8±0.3
2 CFU/25 g (20 h)	17.9±0.7	16.7±0.9	15.7±0.1	17.4±0.8	16.2±0.4	15.3±0.5
2 CFU/25 g (20 h)	17.9±1.0	16.9±0.8	15.7±0.1	17.6±0.6	16.2±0.7	15.7±0.6
20 CFU/25 g (20 h)	19.0±0.8	16.6±0.5	16.0±0.4	18.7±0.6	16.6±0.2	15.0±0.4

Results represent the average of three experiments ± standard deviations

[a] Positive results for $fliC_{h7}$ in uninoculated ground beef samples

and Bagi 2007). Therefore, enrichment in REB at 42°C at 100 rpm allows for relatively rapid growth of *E. coli* O157:H7 in the foods tested, since all samples inoculated with ≤2 CFU/25 g or 25 ml of food were positive after 8 h of enrichment. In most cases, the Cts were lower in samples subjected to 20 h of enrichment compared to 8 h. Overall, the Cts from ground beef and raw milk samples were notably higher than those from lettuce and apple cider samples. Lettuce and cider samples enriched for 20 h generated Cts ranging from 15.3 to 19.0, while those from ground beef and raw milk samples ranged from 20.9 to 28.1.

For assay 2, all results were also positive for samples inoculated with about 2 and about 20 CFU/25 g or 25 ml of food after 8 and 20 h enrichment, and uninoculated controls were negative. However, the ground beef uninoculated controls gave a positive result for the $fliC_{h7}$ target (Table 2; Fig. 1d). Five additional primers sets were designed based on the $fliC_{h7}$ sequence (GenBank accession no. U47614) and tested in the multiplex PCR using uninoculated ground beef enrichments, and results were always positive. Reid et al. (1999) demonstrated evidence of a mosaic gene structure in the H7 *fliC* gene, indicating the exchange of DNA segments within the gene between strains and recent lateral

gene transfer of the H7 *fliC* gene between bacteria with unrelated chromosomal backgrounds. Therefore, it is possible that other bacteria may have acquired regions of the H7 *fliC* gene. In the current study, specificity testing with FLICH7-1068F and FLICH7-1314R using strains having all of the known *E. coli* H antigens generated negative results; therefore, a positive result with the uninoculated samples may either be due to cross-reactivity with genes in non-*E. coli* bacteria found in ground beef, with *E. coli* strains with an H antigen gene not yet unidentified, or with bacteria that may have acquired regions of the H7 *fliC* gene by horizontal DNA transfer. Therefore, the $fliC_{h7}$ gene may not be a suitable target for detection of *E. coli* O157:H7 in ground beef samples. The nature of this cross-reactivity requires further investigation.

The presence of inhibitors of the PCR, such as bilirubin, bile salts, hemoglobin degradation products, polyphenolic compounds, proteinases, complex polysaccharides, calcium, and fat found in food and other complex matrices, and problems with reproducibility have, to some extent, prevented wide acceptance of PCR-based methods for food testing. To address these concerns, a research project, known as FOOD-PCR (http://www.PCR.dk), was approved by the European Commission in 1999 to validate and standardize the use of PCR for detection of pathogens in foods (Malorny et al. 2003). A number of strategies have been employed to minimize PCR problems associated with interfering substances, including filtration, centrifugation, DNA extraction, and the addition of amplification facilitators to the reaction (Al-Soud and Rådström 2000; Rädström et al. 2003). Hoorfar et al. (2004) call attention to the importance of using an internal amplification control as an indicator of PCR failure. They recommended that the internal amplification control should be plasmid DNA carrying a cloned control sequence or purified PCR products and also that the control sequence be detected by sequence-dependent hybridization probes or gel electrophoresis. The 16S rRNA gene of eubacteria was used as an internal control in a PCR assay targeting histamine- and tyramine-producing bacteria, and unmodified pUC19 targeting *rep* of pMB1 has also been used as an internal amplification control (Coton and Coton 2005; Fricker et al. 2007). We investigated the use of three non-competitive internal amplification controls (targeting the *C. jejuni luxS* gene, the pMB1 replicon *rep* in pUC19, and the 16S rRNA gene) in the multiplex PCR assays. All of the multiplex PCR reactions, including the uninoculated controls, generated a positive signal from the internal controls. The probes targeting the internal control sequences were labeled with Cy5, and the probes targeting the *E. coli* O157:H7 genes were labeled with the reporter dyes, FAM (wzy_{O157}), TET (*eae* or $fliC_{h7}$), and Texas Red (stx_1, stx_2). Therefore, in samples positive for *E. coli* O157:H7 a fluorescence signal is generated in all four channels of the SmartCycler. A specific concentration of linearized plasmids containing the *luxS* and pMB1 replicon *rep* targets was added to the PCR assays as internal control templates; however, when the rRNA gene is used as the internal control template DNA, bacterial DNA naturally present in the tested food sample provides the target DNA, and the concentration would thus be variable in different samples. The *luxS* internal control sequence is derived from the *C. jejuni luxS* gene, and it is possible that *C. jejuni* may be found in some food samples; however, *C. jejuni* will not likely grow well in REB under aerobic conditions. The pMB1 replicon *rep* of pUC19 would not normally be found in the tested food sample, and an optimal concentration of target DNA can be determined and added to the PCR assays, which is an advantage of using pUC19. PCR products obtained following multiplex PCR assays of ground beef and lettuce enrichments using the *luxS* and rRNA gene internal controls are shown in Fig. 1c–e.

In summary, the real-time multiplex PCR assays targeting the wzy_{O157}, stx_1, stx_2, and *eae* or $fliC_{h7}$ genes were sensitive and specific and can be used for the detection of ≤2 CFU of *E. coli* O157:H7 in 25 g or 25 ml of apple cider, raw milk, lettuce, and ground beef after 8 h of enrichment in REB. Use of the non-competitive internal amplification control ensures that a failed reaction is not due to inhibition due to food components, problems with instrumentation, or operator error. The assay targeting wzy_{O157}, stx_1, stx_2, and $fliC_{h7}$ may not be suitable for the detection of the pathogen in ground beef since a positive signal was obtained for $fliC_{h7}$ in uninoculated samples. However, if a fluorescent signal is obtained only for the $fliC_{h7}$ target and not for stx_1–stx_2 or wzy_{O157}, it is likely that the sample is not contaminated with *E. coli* O157:H7. The real-time multiplex PCR assays are also being evaluated for testing for *E. coli* O157:H7 in other types of foods and in environmental samples.

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